STAIN TECHNOLOGY

VOLUME VII JULY, 1932 NUMBER 3

PROGRESS IN THE STANDARDIZATION OF BIOLOGICAL STAINS

REPORT ON EXECUTIVE COMMITTEE MEETING HELD AT NEW YORK, MARCH 26, 1932

On calling the meeting to order the Chairman directed attention to two vacancies on the committee, one by the resignation of R. W. French, the other thru the death of W. C. Holmes.

As Mr. French had been vice-chairman, his resignation caused a vacancy in that office and nominations were called for. Only one nomination was made, and the nominee, Dr. S. I. Kornhauser, was promptly elected to the office.

Mr. Herrick, Chemist in Charge of the Color and Farm Waste Division of the Department of Agriculture, who had come in Mr. Holmes' place, announced that the Chemical society would probably appoint him temporarily in Mr. Holmes' position until some one was found to carry on the work in a more active way. Meanwhile, the routine work would be undertaken by a Commission employee, Mrs. A. P. Bradshaw, who had been assisting Mr. Holmes for about two years.

A financial report was submitted by the Chairman showing the following facts:

Total operating expenses for the year $10,830.84, which were met largely by an appropriation from the Chemical Foundation, altho $2,000 were contributed from the Commission's income derived from certification fees, sales of labels and publication profits.

In spite of taking this sum of $2,000 out of the Commission's income, the savings fund of the Commission grew from $4,459.93 to $5,256.06 during the year. This fund is being built up out of surplus income and is intended as the beginning of an endowment.

The two chief items of the Commission's income (disregarding the appropriation from the Chemical Foundation) were: $1,834.92 from sale of labels and from fees for testing stains, $811.87 profits from publications.

The profits on Stain Technology alone for the year 1931 were $552.28.

An amendment to Article III of the Constitution was proposed

covering the method of election of the Chairman. The object of this amendment is to simplify the process, making bi-annual elections unnecessary. By the new amendment, the Chairman holds office until resignation, but with the provision that a new election can always be demanded by three members of the Commission or by one member of the Executive Committee. A copy of this amendment accompanies this report.[1]

Considerable discussion was carried on concerning the advisability of broadening the scope of the Commission. Two lines in particular were discussed. One referred to the study and standardization of hydrogen-ion indicators and other chemicals used in micro-technic; the second referred to the publication of pamphlets dealing with technic in various phases of micro-technic. Both were approved in principle, with the understanding that new undertakings of such nature should be submitted individually to the Executive Committee. In particular the Chairman was authorized to act for the Commission as publishing agent for a periodical service of this nature already established by the Society of American Bacteriologists; namely, the Pure Culture Study of Bacteria. The question was discussed as to the advisability of changing the name of the organization to indicate such broadened activities. This was, however, considered unadvisable, and the matter dropped.

Respectfully submitted,
H. J. Conn, Chairman

[1]The amendment has since been approved by the Commission in a vote taken by mail.

AMENDMENT TO THE CONSTITUTION

Adopted May 15, 1932

The second, third and fourth paragraphs of ARTICLE IV (EXECUTIVE COMMITTEE) shall be amended to read as follows:

CHAIRMAN. The Chairman of the Commission and of the Executive Committee, in office at the time this amendment is adopted, shall continue as Chairman until resignation or until a successor is elected.

An election for the office of Chairman shall be held any year upon the written or spoken request of any member of the Executive Committee or upon written request signed by any three members of the Commission. At the meeting of the Executive Committee at which such request is received, or at the first meeting thereafter if the Committee is not in session at the time of its receipt, a member or two members of said Committee shall be nominated as a candidate or as candidates for Chairman. Within two weeks of said meeting, a ballot shall be mailed to all members of the Commission at their last known addresses, on which shall be printed the name of the candidate or candidates nominated by the Executive Committee and a blank space for writing in the name of any other member of said Committee for which a vote is to be cast instead of for a candidate nominated by said Committee. The polls shall be open from the day of mailing said ballots until 10 o'clock P.M. on the day of election as specified on the ballots; and said day of election shall be at least two weeks after the date of mailing the ballots. The ballots shall be opened by a teller selected by the Executive Committee, who shall declare the person receiving the largest number of votes cast to be elected Chairman. The Chairman thus elected shall take office immediately.

Stains Recently Certified

In the table below is given a list of the batches of stain approved since the last one listed in the April number of this journal.

Stains Certified March 1, to May 31, 1932*

Name of dye	Certification No. of batch	Dye Content	Objects of tests made by Commission†	Date approved
Methylene blue.	NA 6	82%	As histological and bacteriological stain	Apr. 5, 1932
Brilliant cresyl blue.....	NV 11	61%	As vital blood stain	Apr. 6, 1932
Safranin.....	CS 5	85%	As bacteriological stain; not for cytology	May 16, 1932
Carmin.....	LCa 1	——	As histological and cytological stain	May 19, 1932
Acid fuchsin....	NR 1	60%	For use in histology and in the Andrade indicator	May 19, 1932

*The name of the company submitting any one of these dyes will be furnished on request.

†It is not to be inferred that these are the only uses for which each of these samples may be employed. The Commission ordinarily tests each dye for such of its common uses as seem to give the most severe check as to its staining value. Certification does not in any instance, however, imply approval for medicinal use.

WALTER C. HOLMES

1884 to 1932

WALTER C. HOLMES

In Memoriam

Mr. Walter C. Holmes, Research Chemist in the Bureau of Chemistry and Soils, died suddenly on Friday afternoon, March 18th, at Washington, D. C. The news of his death has come as a great shock to his many friends, among whom the members of the Executive Committee of the Commission on Standardization of Biological Stains count themselves.

From 1922 until his death, he zealously devoted his keen mind and unusual experience to the purpose for which the Stain Commission exists. To his industry, good judgment and skill, much of the success of the Commission has been due.

Mr. Holmes was graduated *magna cum laude* from Harvard University in 1906 and was for a few years research chemist in the Technical Laboratory of the DuPont Company at Deepwater, N. J., where he supervised their research methods in color testing and application. He entered the government service in 1922, shortly after the founding of the Biological Stain Commission, while Dr. J. A. Ambler was head of the Color Laboratory of the Department of Agriculture. Mr. Holmes became associated with the latter in the early work of the Stain Commission, and when Dr. Ambler's health forced him to resign a few years later, Mr. Holmes assumed the chief responsibility of the work on Biological Stains carried on at the Color Laboratory. His work in this field consisted largely of the spectrophotometric examination of dyes and their analysis by this method. He was instrumental in developing several new methods of dye analysis which have proved particularly useful in the standardization of stains.

Mr. Holmes, moreover, was internationally known for his work on the synthesis of medicinal dyes. Because of his knowledge along such lines, he was selected by the Rockefeller Institute to collaborate in investigations to which spectrophotography is applicable. He was appointed collaborating expert in the field of absorption spectra of coloring matters for the International Critical Tables.

Mr. Holmes was associate editor of STAIN TECHNOLOGY from the time of its establishment in 1926. From 1929 until his death, he was representative of the American Chemical Society on the Commission on Standardization of Biological Stains, and, accordingly, a member of the Executive Committee of the latter.

The members of this Executive Committee, however, feel that their relationship with Mr. Holmes was more than a mere official tie and that they will miss him as a friend whose personality endeared

him to all who knew him. It is, therefore, the wish of this Committee, incorporated in a motion unanimously adopted at a meeting in New York on March 26, 1932, that a notice be printed in STAIN TECHNOLOGY as evidence of the honor and affection in which Mr. Holmes was held. The following resolutions were therefore drawn up:

In consideration of the untiring zeal and diligence with which the work of the Commission on Standardization of Biological Stains has been forwarded for a period of about ten years by Mr. Walter C. Holmes, and the keenness of mind with which he has attacked the problems of the Commission

We hereby express our deep sorrow that he has been called from our midst, and has thus left vacant a position which we can never hope to fill completely. We shall miss him as a scientist highly trained in his special field, as a member of our Executive Committee whose advice was ever greatly valued, and as a friend whose companionship was always enjoyed.

We do, moreover, extend our sympathy to Mrs. Holmes and at the same time assure her that, altho our loss cannot compare with hers, her grief is shared by ourselves and by all Mr. Holmes' associates.

The Executive Committee
Commission on Standardization of Biological Stains.

HISTORY OF STAINING

The Use of Dyes as Vital Stains

H. J. Conn, *Agricultural Experiment Station, Geneva, N. Y.* and R. S. Cunningham, *Dept. of Anatomy, Vanderbilt University, Nashville, Tenn.*

Vital staining is generally regarded as a recent development. It is undoubtedly entirely correct thus to regard it, as the procedure has been put on a scientific basis only in recent years and its real development does not date back to the year 1900. It must, nevertheless, be recognized that vital staining was practiced much earlier than the staining of dead tissue, unless we take into account the work of Hill (1770) which was discussed in an earlier paper of this series. Hill used dyes for the microscopic study of twigs and in some procedures quite plainly killed the wood before staining, thru the use of such reagents as alum and spirits of lime. Most of the early microscopists, to employ stains, however, applied them directly to the living organisms.

Thus, as discussed in the first paper of this series, we find Ehrenberg (1838) watching the process by which carmin is taken up by infusoria. Also a little later Göppert and Cohn (1849) used carmin for staining the cell contents of living Nitella, while Osborne (1856) studied the coloring of the cell contents of plants grown in colored solutions.

These instances of vital staining, however, were more or less isolated observations and when Gerlach (1858) established the use of stains in histology, their application to fixed tissue so over-shadowed the previous findings in connection with living organisms that the earlier line of work was not further investigated for some time.

This point is well brought out in a discussion of the history of vital staining written by Fischel for Ehrlich's Enzyklopädie der Mikroskopischen Technik and quoted very fully in Krause's 3rd edition of the same. A translation of this historical discussion from Krause (1926) follows:

The history of intravital staining extends, indeed, into far older times than the staining of dead tissue. Even the body colorings which are met in nearly all primitive people really belong in this category. Leaving out of account these practices upon one's own body, a certain industrial and economic impetus has had this method for its object. Thus Pilliet observed that he learned from a conference with Marquis Tseng that the Chinese were already familiar in the

most ancient times with the coloring of living tissue. Naturally, it could not fail to happen that such an interesting observation as the absorption of dyes on the part of living tissue should attract the attention of naturalists. Thus, this method was employed at least as early as the sixteenth century A.D.; M. Lassus (I have Prof. S. Mayer to thank for this information) in his book "Essai ou discours historique et critique sur les découvertes fites en Anatomie par les Anciens et par les Modernes" (Paris 1783) p. 74 makes the statement: "Antione Mizaud, a physician in Paris, observes that madder has the property of reddening the bones of animals feeding on that plant"; the work of Mizaud, he himself cites as follows: Memorabilium utilium et jucundorum centuriae novem., auctore Anton. Mizaldo, Lutetiae 1567; the place cited is found in Cent. 7 Art. 91 Fol. 104. In this use of the property of madder to color during life a newly forming bone, we have the first known beginning of the employment of the vital staining methods from the angle of natural science. This method itself is thus known to have been employed in a productive manner for the study of bone growth as early indeed as the eighteenth century. Besides the studies of Rutherford and Gibson, it was indeed Belchier (1736) and Duhamel (1741–43)[1] who first used it with distinctly successful results that were continuously observed. Of the other authors who reported this method, one should mention Flourens (1842), Serres, and the Germans, Lieberkühn (1874) and Köllicker (1823) (c.f. the statements of Heckel and Giercke). We have K. Gottlieb (1914) to thank in recent times for observing the investigation on the theoretical meaning of the staining of madder. . . .

The methods developed in such a brilliant manner for the staining of fixed tissue owe their development primarily to the sensational (at that time) investigation of Gerlach (1858) on the coloring of tissue with solutions of carmin. These investigations were actually stimulated by means of such studies on intravital staining. These had been carried out on plants, and those of S. G. Osborne (1857) are indeed especially interesting, in which he grew wheat in carmin solutions and observed the staining of the tissue. Gerlach himself tried to stain living animal tissue, but did not obtain any positive results. The increasing success of the staining methods upon dead tissue then placed the use of intravital staining for histological purposes almost entirely in the background for a long period of time. On the other hand, they were in the meantime successfully employed by physiologists. The first interest was directed to this by the decided opinion of Bichat that organic life was represented by two processes, of which one had a destructive effect, the other a constructive effect on the tissue; (in the further development of this idea it was declared that each living being completely replaces itself in seven years and so represents materially a new body). In intravital staining it was believed that a means existed to determine the length of these replacement processes. Magendie opposed this view, calling attention to the fact that the molecule of madder after a

[1]These papers, cited by Krause, have not been located.

definite length of time would be decomposed in the body without bringing about distinct changes of the tissue in which it was contained.

The systematic investigation of the problems in question can best be divided into three groups, of which the first two belong the closer together. The earliest investigations concerned the absorption and distribution of granular material (group 1). Thus to cite an example, Dujardin (1841)[2] found by means of the carmin method that certain ciliated infusoria took up carmin only thru a very definite point, an actual mouth, in contrast to others which absorbed thru their entire surface. Numerous later investigations have further elucidated the phenomenon of "phagocytosis". There followed in recent times the systematic investigation of the intestinal epithelium of all classes of animals (C. H. Jordan 1913). Ponfick, Hofmann and Longerhans investigated the distribution of granular substances within the bodies of vertebrates and furnished a good insight into the mechanism of distribution which already prepared the way for that one which in modern science under the name of the reticulo-endothelial system (L. Ashloff 1925) has brought about much progress in physiology and pathology. Closely related to these studies are the investigations with soluble acid dyes (group 2) whose use has brought nearer to a solution important problems concerning the physiology of secretion and absorption, especially concerning the storage and distribution inside the body. Their introduction is due to the investigations of Chrzonszczewski (1866), R. Heidenhain (1874) and R. Witich (1875). The continuation of these methods was due to the investigations of P. Ehrlich (1885, 1886, 1894), and in recent times especially of H. Ribbert (1904), E. Goldmann (1909, 1912, 1913), L. Ashloff (1925), and the pupils of W. Schulemann, W. v. Mollendorff and others. Today a long series of important theoretical principles of this promising method have been explained, and they have been used in their application to many questions concerning vertebrates. In the case of non-vertebrates, on the other hand, there is still lacking a theoretically satisfactory significance of the nevertheless numerous investigations (Eisig, 1887, Kowalewsky, 1889, P. Mayer, 1882, Metschnikoff, Schindler, 1887, Schneider, 1896, Solger, 1897, and others).

The third group of methods makes use of certain basic dyes and goes back to the work of P. Erhlich. It directed the interest of histologists back to the living cell, assisted by the Altmann theory of cell granulation. In the development of this method O. Schultze, 1887, G. Galeotti, 1884, S. Mayer, 1905, A. Fischel, 1899, 1901, J. Arnold, 1899, E. Herzefeld, 1916, E. Nirenstein, W. v. Moellendorff and others were especially concerned. This method was first systematically applied by Pfeffer to the plant cell, which can not be further considered here any more than can bacteria.

As the above historical discussion does not go into much detail concerning the more modern work on vital staining, or take up much of the development in other countries beside Germany, it is especially

[2]Reference not given by Krause.

interesting to compare it with a recent summary of the same subject from an English source (Cappell 1929). The following quotation from Cappell supplements very effectively the discussion of the subject translated above:

"While there are numerous earlier instances on record in which substances of various kinds have been administered to living animals with a view to demarcating certain cells and tissues, the study of intravitam staining may be said to date from the work of Bouffard (1906) and Goldmann (1909). Previously experiments in vital staining had been carried on chiefly with basic dyes such as neutral red and methylene blue, though in some experiments (Ribbert 1904) lithium carmine, indigo carmine and various suspensoids had been used. The staining of the osseous system by feeding madder to growing animals has also been known for many years, and has long been used to demonstrate areas of new osseous deposition.

"Goldmann studied in detail intravitam staining with acid colloidal dyes. Working under Ehrlich's guidance, he was the first to describe the distribution of vital stains in the animal body after repeated injections, and he elaborated methods which secured extremely high degrees of staining. In these experiments pyrrhol blue was mainly used, as after extensive observations this was found the most satisfactory dye. In his later work Goldmann used also isamine blue—a dye of similar constitution—but he frequently stated that no dye was as good as his original pyrrhol blue. In our own experiments, this difficulty has been experienced and none of the samples of isamine blue or pyrrhol blue used have given such intense and selective results as in Goldmann's earlier work. It seemed to Goldmann that vital staining offered the prospect of a further insight into cell physiology. He regarded the stained granules as evidence of secretory activity on the part of the cells in which they appeared and sought to identify them with other known cell constituents. He recognized that while many cells of widely differing types were capable of exhibiting vitally stained granules in varying degree, yet the dye appeared predominately in certain elements which he named 'pyrrhol' cells. Many of these are wandering mononuclear cells of the tissues, and Goldmann investigated the reaction of such pyrrhol cells in diseased conditions. At all times, however, he retained the view that capacity for vital staining was intimately connected with cellular secretory activity. While most recent authors differ from Goldmann in the interpretation of the phenomena, the accuracy of his description of the appearances is unquestioned. Shortly after Goldmann's work there appeared that of Kiyono (1914), on the results of an extensive series of experiments with lithium carmine and certain other vital stains.

"Since 1914, there have been many papers dealing with different aspects of the problem. We shall deal here only with the most important publications concerning each branch of the subject. Evans and Schulemann (1915) claim that the essential phenomenon of vital staining, i.e. generalized staining of the tissues together with the

appearance within the cells of granules stained by the dye, can be explained in terms of physical chemistry, and that the effectiveness of a dye as a vital stain depends on the colloidal state of its solution, and is independent of its chemical constitution. According to this view, when the solution is in a suitable colloidal state it accumulates as granules within the cytoplasm of the cells. These authors have dealt at great length with this aspect of the problem and their work will be referred to more fully when the nature of vital staining is considered.

"The study of vital staining in tissue cultures, especially by the Lewises (1915), has yielded information concerning chiefly the nature of the vacuoles and granules which become visible after application of the stains and their relation to other cell constituents. Mitochondria have also been studied by supravital staining and this method is stated to be the most satisfactory for demonstrating these elements (Michaelis 1900; Bensley 1911; and Cowdry 1918).

"The knowledge obtained from observations on normal vitally stained animals raised great hope that by means of the criteria which demarcated certain very clearly defined groups of cells, one could follow the activity of these cells in inflammatory and other reactive processes. Thus in recent years a great mass of literature has appeared dealing with the study of problems of pathology by methods of intravitam staining. Maximow (1902, 1927) and his co-workers have studied the relation of the 'wandering connective tissue cells' of the body to other cell types; McJunkin (1919) and Foot (1920) have investigated the relations of the vascular 'endothelial cell' to the elements of the blood and to the wandering cells of connective tissue; the researches of Sabin (1923, 1924) and her co-workers have dealt with the specificity of the cellular elements of the blood, lymph and serous sacs.

"In the field of experimental embryology, vital staining has been used to demonstrate the origin and mode of growth of the lymphatics in amphibian larvae (Wislocki 1917). Goldmann observed that in rats and mice vital staining of the mother was not transferred to the foetus, altho the membranes and placenta became deeply stained. His observations have been generally confirmed by Wislocki (1920, 1921) in guinea-pigs, rabbits and cats, and by Shumidzu (1922) in rats and mice, but in the rodents, Wislocki found that a very small amount of dye might be transmitted to the amniotic fluid and foetus producing a faint staining; no trace of such staining ever appeared in cats. Wislocki has further shown (1921) that soluble dyes injected directly into the amniotic sac are absorbed into the foetal circulation and bring about vital staining of the foetus, but no evidence of absorption in the maternal circulation could be found."

Neither of the two papers so liberally quoted above pays much attention to the staining of living leucocytes. This subject was treated very briefly in an earlier article of this series, but in so few words that a more detailed discussion seems called for here.

Ranvier (1875) observed that a frog's leucocytes could engulf

particles of carmin which could be seen within them as they passed thru the walls of capillaries. The first biologist, however, to use stains in the actual study of leucocytes was Certes (1881) who employed cyanine and quinolene blue for the differential staining of frog's leucocytes and noticed that they retained their motility for a short time, while taking up the dye. Galleotti (1894) used a considerable series of dyes similarly, and apparently was the first thus to employ neutral red, which now has such wide application as a vital stain for blood. His work, however, was principally on the salamander, *in toto*, and considered the blood only incidentally. Pappenheim (1895) and Isreal and Pappenheim (1896) developed the neutral red technic much further, adapting it to the study of young red blood cells and thus initiating the present methods for applying dyes to the study of reticulation and basophilia. Arnold (1899, 1900) reported a careful study of the granules of living leucocytes, describing the changes occurring in both nucleus and cytoplasm after various periods of exposure to neutral red and methylene blue.

At about this same time, a very important advance in the method of applying dyes to living blood was made by Uhma (1899) when he showed how to employ neutral red in alcoholic solution. Previous workers in this field had avoided destruction of the cells thru osmotic action by using dyes dissolved in normal salt solution or by adding a crystal of dye directly to the blood. Uhma, on the contrary, dissolved the dye in alcohol and allowed this solution to dry on the clean slide. Thus a very thin layer of stain was precipitated on the slide. When the drop of blood was applied to the slide, the precipitated dye was distributed evenly thru it, without changing the concentration of the electrolyte in which the cells were suspended. Uhma used this method for the study of gonococci in pus, but also noted that the granules of the leucocytes, which were present in the pus, were stained yellow. Pappenheim (1899) in the same year described almost identically the same method of applying various dyes (inincluding neutral red) for the staining of blood. Nakanishi (1900) followed Uhma's technic, applying it to the study of bacteria, protozoa and blood cells. The method thus introduced by Uhma and by Pappenheim has been in general use since that time.

Other workers have developed methods for staining leucocytes in the tissues. Thus Maximow (1906) stained them in diffuse connective tissues, while Dubreuil (1913) used neutral red for living cells from the peritoneal cavity.

A new impetus was given to the subject in the early '20's by the work of Simpson (1921, 1922) and Sabin (1921, 1923). The former

studied the reactions of living leucocytes to over two hundred dyes; she was concerned primarily with the question of segregation vacuoles but also mentions specific granules (of the granulocytes), lipoid granules and mitochondria. Dr. Sabin's earlier paper discussed the differentiation of blood cells in embryo chicks by the use of neutral red, while her later paper contains the first thoro analysis of human blood cells made with supravital stains. Her important contributions in this field were made with the dyes neutral red and Janus green B.

Since the publication of Sabin's study of living leucocytes, many papers have appeared dealing with pathological changes in various types of blood cells, but no further effort was made in regard to careful description and illustration of normal cells until Cunningham and Tompkins (1930) described the supravital staining method in considerable detail, publishing colored illustrations of normal human cells[3].

BIBLIOGRAPHY

Arnold, J. 1899. Über Granularfärbung lebender und überlebender Leukocyten. Arch. Path. Anat. & Phys., **157,** 424–437.

1900. Über Granula-Färbung lebender und überlebender Gewebe. Arch. Path. Anat. & Phys., **159,** 101–116.

Aschoff, L. 1925. Das reticulo-endotheliale System. Erg. inn. Med. & Kinderh. Berl., **26,** 1–118.

Bensley, R. R. 1911. Studies on the pancreas of the guinea pig. Am. J. Anat., **12,** 297–388.

Bouffard, G. 1906. Injection des couleurs de benzidine aux animaux normaux. Ann. Inst. Past., **20,** 539–546.

Cappell, D. F. 1929. Intravitam and supravital staining. J. Path. & Bact., **32,** 595–707.

Certes, M. A. 1881. Sur un procédé de coloration des Infusiores et des éléments anatomiques, pendant la vie. Compt. Rend. Acad. Sci., **92,** 424–426.

Chrzonszczewsky, N. 1866. Zur Lehre der Lungenepithel. Arch. Path. Anat., **35,** 165–168.

Cowdry, E. V. 1918. The mitochondrial constituents of protoplasm. Contrib. to Embryo. (Carnegie Inst.) **8,** 41–160.

Cunningham, R. S. and Tompkins, E. H. 1930. The supravital staining of normal human blood cells. Fol. Haematol., **42,** 275–270.

Dubreuil, G. 1913. Le chondriome et le dispositif de l'activité sécrétoire. Arch. d'Anat. Micr., **15,** 53–151.

Ehrenberg, C. G. 1838. Die Infusionsthierchen als volkommene Organismen. Leipzig.

Ehrlich, P. 1885. Sauerstoffbedürfnis des Organismus. Berlin.

1886. Über die Methylenblaureaction der lebenden Nervensubstanz. Deutsche Med. Wochnsch., **12,** 49–52.

[3]An additional note on the preservation of vitally stained material will appear in the next number of this Journal.

1894. Über Neutralroth. Alg. Med. Centbl., 1894, No. 2, 20. *Abs. in* Zts. Wis. Mikr., **11,** 250.

EISIG, H. 1887. Monographie der Capitelliden des Golfes von Neapel. Fauna Flor. Golf Neapel, **16,** 1–906.

EVANS, H. M. and SCHULEMANN, W. 1915. Über Natur und Genese durch saure Farbstoffe entstehenden Vitalfärbung granula. Fol. Haematol., Archiv., **19,** 207–219.

FISCHELL, A. 1899. Über vitale Färbung von Echinodermeneiern während ihrer Entwickelung. Anat. Hefte, I. Abt., **11,** 461.

1901. Untersuchungen über vitale Färbung. Anat. Hefte, I. Abt., **16,** 417–519.

FLOURENS, J. P. M. 1842. Recherches sur le developpement des os et des dents. Gide, Paris.

FOOT, N. C. 1920. Studies on endothelial reactions. J. Exp. Med., **32,** 513–546.

GALEOTTI, G. 1894. Recherche sulla colorobilita della cellule viventi. Zts. Wis. Mikr., **11,** 172–207.

GERLACH, J. 1858. Mikroskopische Studien aus dem Gebiet der menschlichen Morphologie. Erlangen.

GOLDMAN, E. E. 1909. Die äussere und innere Sekretion des gesunden Organismus im Lichte der vitalen Färbung. Beitr. z. Klin. Chir. Tübingen, **64,** 192.

1912. Die äusere und innere Sekretion des gesunden und des kranken Organismus. Id. **83,** 1–108.

1913. Vitalfärbung am Zentralnerven System. Reiner, Berlin.

GÖPPERT, H. R. and COHN, F. 1849. Über die Rotation des Zellinhaltes von Nitella flexilis. Bot. Zeitg., **7,** 665, 681, 697, 713.

GOTTLIEB, B. 1914. Die vitale Färbung der Kalkhaltigen Gewebe. Anat. Anz., **46,** 179–194.

HERZFELD. 1916. Über die Natur der am lebenden Tier erhaltenen granularen Färbungen bei Verwendung basicher und saurer Farbstoffe. Anat. Hefte. **54,** 451–523.

HILL, J. 1770. The construction of timber, from its early growth; explained by the microscope, etc. London.

HEIDENHAIN, R. 1874. Mikroskopische Beiträge zur Anatomie und Physiologie der Nieren. Arch. Mikr. Anat., **10,** 1–50.

HOFFMAN and LANGERHANS. 1869. A. Path. Anat. (Cited from Krause, Original cannot be located.)

ISREAL, O. and PAPPENHEIM, A. 1896. Über die Entkernung der Saugethiererythroblasten. Arch. Path. Anat. & Phys., **143,** 419–447.

JORDAN, H. 1913. Vergleichende physiologie wirbelloser Tiere. Vol. 1. G. Fischer. Jena.

KIYONO, K. 1914. Die vitale Karminspeicherung. Jena.

KÖLLIKER, ALBERT v. 1823. Die normale Resorption des Knochengewebes und ihre Bedeutung für die Entstehung der typischen Knochenformen. Vogel. Leipzig.

KOWALEWSKY, A. 1889. Ein Beitrag zur Kenntnis der Exkretionsorgane. Biol. Zentbl., **9,** 33–47, 127–128.

KRAUSE, R. 1926. Enzyklopädie der Mikroskopischen Technik. Vol. I. Berlin. (See pp. 697–721.)

LEWIS, M. R. and LEWIS, W. H. 1915. Mitochondria (and other cytoplasmic structures) in tissue cultures. Am. J. Anat., **17,** 339–401.

LIEBERKÜHN, N. 1874. Über ein Einwirkung von Alizarin auf die Gewebe des lebenden Körpers. Gesellsch. Naturw. z. Marburg, Sitzungsber. **1874,** 33–46.

MAC JUNKIN, F. A. 1919. The origin of the phagocytic mononuclear cells of the peripheral blood. Am. J. Anat., **25,** 27–53.

MAXIMOW, A. 1902. Experimentelle Untersuchungen über die enzündliche Neubildung von Bindegewebe. Beitr. z. Path. Anat. & z-allg. Path. 1902. Suppl. 5, 1–258.

1906. Über die Zellformen des lockeren Bidegewebes. Arch. Mikr. Anat., **67,** 680–757.

1927. Morphology of the mesochymal reactions. Arch. Path. & Lab. Med., **4,** 557–606.

MAYER, P. 1882. Der Caprilliden des Golfes von Neapel. Fauna Flora Golf Neapel, **6,** 1–197.

MAYER, S. 1905. Prag. Med. Wochensch., **47,** 187. (Original not seen. Cited from Krause, 1926.)

METCHNIKOFF, E. 1902. Cited in Krause, 1926. Probably refers to a German translation of L'immunitié dans les maladies infectienses IX. Masson, Paris, 1901.

MICHAELIS, L. 1900. Die Vitale Färbung, einer Darstellungsmethode der Zellgranular. Arch. Mikr. Anat., **55,** 558.

1905. Ultramikroskopische Untersuchungen. Arch. Path. Anat. & Physiol., **179,** 195–208.

NAKANISHI, K. 1900. Vorläufige Mittheilung über eine neue färbungsmethode zur Darstellung des feineren Baues der Baterien. Münch. Med. Wochnsch., **47,** 187–8.

OSBORNE, S. G. 1857. Vegetable cell-structure and its formation as seen in the early stages of the growth of the wheat plant. Trans. Micr. Soc., **5,** 104–122.

PAPPENHEIM, A. 1895. Die Bildung der roten Blutscheiben., Inaug., Dissert. Berlin.

1899. Vergleichende Untersuchungen über die elementare Zummansetzung des rothen Knochenmarkes einiger Säugethiere. Arch. Path. Anat. & Phys., **157,** 19–76.

RAVIER, L. 1875. Traité technique d'histologie. Savy, Paris.

RIBBERT, JUGO. 1904. Die Abscheidung intravenös injizierten gelösten Karmins in den Gewbeen. Zts. Allg. Physiol., **4,** 201–214.

SABIN, F. R. 1921. Studies on blood. The vitally stainable granules as a specific criterion for erythroblasts and the differentiation of the three strains of the white blood cells as seen in the living chick's yolk-sac. Bul. Johns Hop. Hosp., **32,** 314–321.

1923. Studies on living human blood cells. Bul. Johns Hop. Hosp., **34,** 277–288.

SABIN, F. R., AUSTRIAN, C. R., CUNNINGHAM, R. S., and DOAN, C. A. 1924. Studies on the maturation of myelocytes and on amitotic cell division in the peripheral blood in subacute myeloblastic leucemia. J. Exp. Med., **40,** 845–871.

SCHINDLER, E. 1878. Beiträge zur Kenntnis der Malpighi 'schen Gefässe der Insecten. Abs. in Zts. Wis. Zool., **30,** 587–658.

SCHNEIDER, G. 1896. Über phagocytäre Organe und Chloragogenzellen der Oligochäten. Zts. Wis. Zool., **61,** 363–392.

SCHULZE, O. 1887. Die vitale Methylenblaureaktion der Zellgranula. Anat. Anz., **2,** 684–688.

SHIMIDZU, Y. 1922. On the permeability to dyestuffs of the placenta of the albino rat and the white mouse. Am. J. Physiol., **62,** 202–224.

SIMPSON, M. E. 1922. Vital staining of human blood with special reference to the separation of the monocytes. Univ. Cal. Publ. Anat., **1**, 1–9.

1922. Vital staining of human and mammalian blood, with special reference to the separation of the monocytes. Anat. Rec., **23**, 37.

SOLGER, B. 1897. Über vitale Farbstoffimprägnation. Deut. Natf. Verh., **2**, 2nd half, 237.

UHMA. 1899. Die Schnellfärbung des Neiser'schen Diplococcus in frischen nicht fixierten Präparaten. Arch. Dermatol. & Syphilis, **50**, 241–242.

WISLOCKI, G. B. 1917. The staining of amphibian larvae with benzidine dyes with especial reference to the behavior of the lymphatic epithelium. Am. J. Physiol., **42**, 124–132.

1920. Experimental studies on fetal absorption. Contr. to Embryol. (Carnegie Inst.), **11**, 45–60. No. 274.

1921a. Further experimental studies on fetal absorption. Contr. to Embryol. (Carnegie Inst.), **13**, 91–101. No. 276.

1921b. Experimental studies on bone marrow. Bul. Johns Hop. Hosp., **32**, 132–4.

v. WITTICH. 1875. Beiträge zur Physiologie der Nieren. Arch. Mikr. Anat., **11**, 75–93.

THE USE OF BASIC FUCHSIN IN PLANT ANATOMY[1]

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Abstract.—**A method useful in tracing the vascular bundles of herbaceous plants and the water conducting system of woody plants is presented. A stock solution of 2.5% basic fuchsin in 95% alcohol is prepared. A mixture of 1 to 2 parts of the stock solution to 100 parts of tap water (slightly alkaline) is made, standing 12–48 hours and being filtered before use. Cut ends of the living plants to be examined are immersed in the working solution until the dye appears in the parts desired.**

The material may be examined by serial, hand sections mounted in 50% to 75% glycerin, or it may be split, laid open, and examined with a low power lens. Clearer views may be procured by dehydrating in an alcohol series and clearing in methyl salicylate. Permanent mounts may be made by removing the methyl salicylate with xylol and mounting in balsam. Structures such as leaves, which contain considerable chlorophyll, should be first boiled in *slightly alkaline* 85% alcohol until the green color is removed.

Gourley[2] has called attention to the use of basic fuchsin in tracing the vascular bundles of seedling plants. The present writers have extended the usefulness of this dye in their own work to more mature and even woody plants, and present the various technics developed with the hope that other workers may find them of some use.

This particular dye has been chosen because it is relatively stable and is easily carried by the transpiration stream; it gives a brilliant stain, and except in extreme cases, diffuses but little from the water conducting vessels. It is also less toxic than many other dyes.

Considerable trouble was experienced in the beginning due to the wilting of many of the plants before the dye had penetrated the entire vascular system. This wilting was apparently due to a mechanical clogging of the water conducting vessels by precipitated dye. The difficulty was eventually almost completely eliminated by filtering the solution before use. The writers made several hundred observations in numerous carefully controlled experiments to determine the best dye concentrations in combination with both distilled and tap

[1]Paper from the Department of Botany, The Ohio State University, No. 294.

[2]Gourley, J. H. Basic fuchsin for staining vascular bundles. Stain Techn., 5, 99–100. 1930.

(alkaline) water. They also tried various methods in observing the material thus stained, and present the following summary as the best and most simple general technic found for the various woody and herbaceous species studied.

Procedure

Stock Solution. 2.5% solution of basic fuchsin[3] in 95% alcohol.

Working Solution. Mix 1 or 2 parts of the stock solution with 100 parts of tap (alkaline) water. This solution should stand 12 to 48 hours and be filtered before use.

For leaves, twigs, branches, etc., cut under water, if possible, and transfer to the working solution. For whole plants, wash the soil from the roots, cut the ends of the primary and secondary roots under water and transfer to the solution. It is best to place the plants in a well lighted situation, but not in direct sunlight. The transpiration stream carries the dye with it, the dye staining the lignified portions of the vessels.

In examining a plant for the root-shoot transition, do not wait for the dye to show in the leaves, for if this is done the roots will be too heavily stained.

Examination of Material. Fresh serial, hand sections may be placed on a slide moistened with 50% to 75% glycerin. The material may be dissected under a binocular. It may also be split or laid open with a sharp knife and then cleared.

Owing to the stability of the dye and its partial fixation in the lignified elements of the water conducting vessels, material may be easily cleared with but little loss of dye. If the materials, such as seedling plants and mature leaves, contain large amounts of chlorophyll, this must be removed by boiling the specimens in *slightly alkaline* 85% alcohol. They are then passed thru 95% and 100% alcohols and into some clearing agent, generally an "oil". Xylol may be used and is inexpensive. Bergamot and clove oil may also be used, but are more expensive. The writers have found the most effective clearing agent to be synthetic oil of wintergreen (methyl salicylate). Its advantages are: (1) it is an excellent clearing agent, (2) it will clear from 95% alcohol if the material is not too bulky and (3) it is not prohibitively expensive.

Permanent Mounts may be made from thin materials such as leaves, stem sections, etc., by removing the methyl salicylate with xylol (5 minutes) and mounting in balsam. In this way, excellent mounts

[3]While most of this work was done with dye supplied by the Coleman and Bell Company, dye from several other sources also proved satisfactory.

showing the complete ramification of the water conducting system of leaves have been prepared, the small purple stained spiral vessels of the "vein islets" standing out in bold relief against the translucent, but easily identified, mesophyll cells.

Large Trees have also been injected by the writers with considerable success. In this method small holes (⅛ to ¼ inch) were bored near the base and half way thru the trunk. The bit was removed under a jet of water and the delivery tube for the dye immediately put into the hole and clamped into place. The dye was introduced under a slight gravity pressure. While it is not the purpose of the present paper to indicate the results obtained by this method, it may be noted that when the trees were cut the path of the dye was clearly marked, even into the smallest twigs 8 meters and more above the point of injection.

The advantages of basic fuchsin over other dyes for this general type of work may readily be seen to lie in its specificity for the water conducting vessels of plants, due to the fact of so little diffusion of the dye into the surrounding tissues, and in addition the ease with which the material may be cleared and critically examined.

THE MARCHI METHOD FOR DEMONSTRATING DEGENERATED FIBER CONNECTIONS WITHIN THE CENTRAL NERVOUS SYSTEM

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ABSTRACT.—**The routine methods of the above laboratory are given for this technic as well as the most important modifications. Time periods are given and an attempt has been made to review the best explanations of the principles underlying the steps of the method. A discussion of frequently found artifacts finds many of these avoidable and offers suggestions to aid in the interpretation of others.**

Migration of the Marchi particle is explained on the basis of phagocytosis.

Of the methods available for the study of degenerated fiber connections, the *Marchi method*, (Marchi 1897) especially when used in conjunction with some other myelin sheath preparation (usually the Weigert method or one of its modifications) has achieved a valued place in experimental work upon the nervous system.

The procedure depends upon the fact that when a nerve has been injured it undergoes what is known as Wallerian degeneration which includes decomposition of the myelin sheath. With the Marchi method it is possible to stain these changed nerves a black color. The *theory of the processes involved* is not altogether complete.

It has been customary in this laboratory to carry out the method on extirpation specimens, the tissues of which are ordinarily removed 11 days after operation (30 days for retrograde degeneration). After preliminary hardening in 10% neutral formaldehyde (made up from a solution of neutral formalin [formaldehyde 37%]) (24 hours) they are then cut into parallel slices 4 mm. in thickness and removed directly to an abundance of 3% potassium dichromate aqueous solution which has been aged for three months, but which has not been reduced to a green color. In this solution the tissue remains for two weeks and is then placed in about four times its own volume of the Marchi mixture which is made up by equal parts of 3% potassium dichromate and a 1% aqueous solution of tetroxide of osmium (customarily called osmic acid). The time of action of this solution depends upon the size of the tissue, varying from five to fourteen days. The Marchi fluid is examined from time to time and more osmium is added if that which was in the solution is used up. One determines this by cautious smelling. (Even traces of osmium have

a distinctive odor easily differentiated from the musty odor of old dichromate solutions. Caution must be observed since osmic acid fumes are poisonous to the nervous system.) After the tissue has remained in the Marchi solution the requisite time, it is washed in running water for 24 hours and then embedded in celloidin. The sections are usually cut dry at about 40 microns, stored in 50% alcohol and after dehydration are cleared in carbol-xylol and mounted in carbol-xylol-balsam.

We have intimated above that the Marchi method is one based upon many empirical concepts, and it is safe to say that in its use, as in no other histological method, is experience both in preparation and interpretation necessary.

The phenomena of degeneration and regeneration of nerves began with "Steinbruch 1838, Masse 1839, Günther and Schön 1840, but only acquired significance after the discoveries of Augustus Waller in 1852." More recently Ranvier (1882) and in 1928, Cajal, investigated the matter. Injured nerves undergo a distal decomposition which affects the myelin sheath most profoundly, and it is the products of this decomposition with which the osmium reacts. Osmium is immediately reduced by unsaturated fats to black reduction compounds and combines with many saturated fats to undergo subsequent reduction if these fats are affected. Osmium tetroxide is reduced to osmium oxide (OsO_2) by oleic acid and its compounds which are thereby stained black. Palmitic and stearic compounds are stained when exposure to osmic acid is followed by treatment with alcohol which changes it to osmium hydroxide—$Os(OH)_4$. Osmic acid will stain normal myelin sheaths and was the earliest form of myelin stain used. Wlassak (1898) states that pure osmic acid colors fat plus lecithin, but that the Marchi mixture colors only fat. In certain of the old osmic-myelin stains (Exner's method and its modifications) the tissue was fixed in Müller's fluid or potassium dichromate and treated with osmic acid which was subsequently reduced by pyrogallol, and the tissues differentiated by the Pal method. (We have found potassium permanganate followed by a mixture of oxalic acid and sodium sulfite to allow nuclear counter stains).[1] It is believed that the differential staining of the Marchi

[1]Heller (1898) gives a description of a method for staining myelinated fibers as follows:

Fix in Müller's fluid and embed in celloidin. After cutting, the sections are placed in 1% osmic acid at room temperature. Rinse with water and reduce with pyrogallol. (Heller used ordinary photographic developer). Decolorize in potassium permanganate and get rid of the resulting brown color with a 2% solution of oxalic acid. Counterstain if desired.

method is due to the fact that the reducing action of the myelin sheaths has been satisfied by the use of potassium dichromate or chromic acid. From the foregoing, it would seem that in the osmium reactions we have a highly oxidized and unstable material (osmium tetroxide) which is not only readily reducible but possesses considerable power of adherence and will remain in the unreduced state in most materials, particularly those treated with oxidizing agents, but subsequently undergoes ready reduction if some reducing substance appears. In the Exner method the myelin absorbed the osmic acid in the unreduced form. After treatment with pyrogallol, the reduced osmium comes into stable relation with the myelin. (In the Exner method the sections turn completely black, but all the tissue except the myelin loses the black color in the subsequent oxidation of the differentiation process). In the Marchi method the degenerated myelin sheaths undoubtedly possess radicles having reducing power. While it is probably true that the normal myelin may be protected by the previous treatment with potassium dichromate or some other oxidizing substance (Busch 1898) it may also be equally true that it is the presence of such a substance in the Marchi mixture rather than previous treatment which is most important (Wlassak, 1898). This seems to be borne out by the work of Ciaglinski (1896) who eliminated the Müller's fluid and by the practice now occasionally employed of using Benda's or Fleming's solutions (which are chromic-acetic-osmic acid mixtures) upon small pieces of tissue placed directly into them for one or two weeks after preliminary fixation for one day in formalin. Such solutions give results similar to the Marchi fluid but are not as dependable. Mallory and Wright (1924) attribute the protection of the normal myelin to the presence of chrome salts rather than oxidation.

Müller's fluid is the classical fixative for the tissues after removal. The time for the action of this fluid varied considerably from five days to three weeks tho eight days has been usually taken as the desideratum. Singer and Münzer (1898) fixed for this length of time "in toto". Pellizzi (1895) preferred a longer time. While a long time probably does no harm to the staining reactions, it will interfere with the cutting qualities of the brain. In any event the fluid must be frequently changed and precipitates or a greenish color should be avoided. The use of formalin as a preliminary fixative has come into

We found much better results with a weaker osmium bath and a long immersion time.

Certain of the early osmium methods for myelin do not depend upon a preliminary treatment with Müller's fluid.

use and has had vehement defenders and antagonsists. Heilbronner (1898) claims that it causes normal fibers to stain, while Luithleu and Sorgo (1898) feel that it prevents degenerated fibers from taking the stain. Weigert (1897) is of the former opinion, while Mallory and Wright feel that formalin should not be used for finer work. No doubt formalin of inferior grade will produce distorted results, this being especially true of highly acid formalin. In the use of neutral formalin, we have experienced no untoward results and it is probably better in the case of large tissues to use formalin as a preliminary fixative than to depend upon the slow infiltration of Müller's fluid. Parsons (1902) believes it is necessary to be rid of all traces of formalin before placing the tissue in the osmic acid mixture. His routine was to wash the tissue for one day in running water. It is to be noted that he transferred his tissues directly to the Marchi fluid without further treatment with dichromate. Under such circumstances, it is probably advisable to remove the formalin by washing. If, however, the tissue is to stay in Müller's fluid for a while before being placed in the Marchi mixture washing is probably unnecessary. As a substitute for Müller's fluid 3% potassium dichromate is widely used and apparently gives comparable results. Tho small tissues may be passed directly into the Marchi solution from the dichromate, it is best for the sake of thoro impregnation to slice the material. While some cut the tissues into slices 4 mm. in thickness before the use of Müller's fluid, it is probably better to wait until after thoro fixation has occurred. J. Starlinger (1899) has devised a machine for cutting exactly parallel brain slices. We have found a home-made vulcanite mitre box to answer very well for the purpose.

Some investigators transfer the tissues directly to the Marchi fluid without washing. It is probably best to rinse off the slices with distilled water and to make sure that no chromium precipitate is carried over into the Marchi fluid. This fluid is variously made up of equal parts of 1% aqueous solution of osmium tetroxide and Müller's fluid (or 3% potassium dichromate) or as usually prepared, one part of the former to two parts of the latter, Singer and Münzer (1898). Orr (1900) added one part of 1% acetic acid to 4 parts of osmic acid (which latter is used in 2% solution). As originally described, he omitted potassium dichromate, but we notice that in 1902 he always added it to the above mixture. This process he claims hastens impregnation and records the permanence of the stain for two years. Vassale used 3 parts of Müller's fluid to one of the 1% aqueous osmium tetroxide and added 20 drops of nitric acid to every 100 cc. Van Geheuchten uses four parts to one. Weigert (1897) calls atten-

tion that "thin slices of tissue and an abundance of the Marchi fluid insure the accuracy of the method." While in this fluid, care must be taken to prevent the slices from covering each other for the penetration power of the solution is poor. The solution should be changed whenever it blackens. The time of action is variously given from five to ten days. It is said that a longer time does no harm altho the contention that an overstay will produce undue blackening is probably not unfounded. It is probable that long stays in the Marchi fluid will impair the cutting properties of the tissue and render it brittle. Hamilton (1897) calls attention to a condition which he calls "case hardening" due to strong solutions of the Marchi mixture. Such solutions produce a hard black rind on the outside surface of the tissue preventing proper impregnation and causing cracking of the sections.

In the preparation for embedding, the tissue should be very thoroly washed in running water. Twelve to twenty-four hours have been recommended. We have noticed no untoward results after two days of washing.

Almost without exception, the paraffin method is avoided because of the action of its solvents upon fatty products. With small pieces it is occasionally used in its chloroform modification. The tissue should be embedded under such circumstances within 12 to 16 hours. Most frequently the celloidin method is used and is generally satisfactory. The steps should be shortened as much as possible. In this connection it may be mentioned that the use of slightly more than 50% of absolute alcohol in the dissolved celloidin is beneficial in that alcohol protects the osmium against the solvent action of the ether. Langley and Anderson (1899) embedded in gum. The same authors used counterstains. Of these, carbol-fuchsin, safranin, or van Gieson's stains may be employed. Altho paraffin sections may be cut quite thin, sections for tract work are best cut at 40 microns or even greater thickness.

The choice of a clearing agent is a matter of considerable concern, "Osmium which has been reduced by fat is exceedingly soluble in xylol, ether and turpentine, less so in alcohol, chloroform, oil of cloves and oil of origanum" (Mallory and Wright, 1924). Colossak (1898), found petroleum ether followed by vaseline mounting to be of more value than chloroform. We have found carbolxylene of use tho its solvent action soon becomes apparent. The same is true of chloroform, tho the preparations last somewhat longer than with carbolxylene. Butyric alcohol followed by euparal is recommended by Kings-

bury (1927). With castor-xylene, benzene, oil of bergamot, cedar oil, chloroform, and carbolxylene the results were as follows:

Solvent action of carbol-xylene manifested itself after 2 weeks
Solvent action of chloroform manifested itself after 6 months
Solvent action of castor-xylene manifested itself after 6 weeks
Solvent action of benzene manifested itself after 6 months
Solvent action of cedar oil manifested itself after 8 weeks
Solvent action of oil of bergamot manifested itself after 4 weeks

Obviously, the solvent of the balsam will act upon the section. It is best to use hot balsam with the addition of no solvent.

Thruout the processes of the method, attention should be directed to the volatility and chemical instability of osmium tetroxide. Solutions should be made up in scrupulously clean, glass-stopped bottles the stoppers of which have been anointed with an inert grease and kept in a cool place, in complete darkness. The presence of traces of organic matter will blacken the solution. It is best reoxidized under such circumstances by the addition, drop by drop, of hydrogen peroxide solution. Metals should not be introduced into osmium solutions.

Heilbronner (1898) has called attention to the natural safeguards of the Marchi method, but the production of artifacts during the unfixed stages of the brain are exceedingly prevalent (Stronsky, 1903). Normally, the degenerated fibers appear black because of droplets of blackened oil in their sheaths and black varicosities along the sides of the sheaths. Occasionally, such black products occur as flakes. Traumatism of myelinated tissue during the life of the animal is demonstrated in this way till about three months have passed. The maximum time for small mammals is given as 11 days (Allen, 1923), for larger mammals up to the 25th day (McClung, 1929), and for cold blooded animals 30 to 40 days (Kingsbury, 1927). Such traumatism may occur as the result of section of a nerve or compression, Hoche (1896). Singer and Münzer (1898) called attention to a blackening along nerves which could not have been injured prior to removal of the tissue. These and other facts lead one to believe that injury during removal, or at any time before the tissue is completely fixed, will cause the artificial deposition of black particles at the injured point. They listed several focal points for such artifacts which were—the cut surface of any nerve and such a nerve for about ½ cm. back from that cut surface, the cut surface of the spinal cord, the entrance of the posterior root into the posterior nuclei, the arcuate fibers of the medulla and its raphe, the entrance of certain of the cranial nerves particularly the oculomotor and trochlear. Teljatnik

(1897) by means of the Lustgarten-Pal differentiation caused the granules of post-mortem squeezed nerves to disappear. He concluded that they differed from the ordinary fatty products of degeneration. Weigert (1897) calls attention to the fact that one should regard only significant and not isolated, sparse degeneration. Rosin (1896) in a study of the ventral horn of the cord found artifacts in certain of the cells located there.

Boyce (1895) denies that certain tracts or nerve roots normally give the osmium reaction, and indeed it is our belief that under the best conditions of technic this is true, for we have obtained very many preparations in testing this point which showed absolutely no osmium deposition except along the optic tracts and preparations which showed strictly unilateral degenerations. We feel that the deposition we have found so frequently in the optic tract is due to some technical feature in removing the brain. Handling a brain excessively, squeezing it, or putting it in the hot sun will cause peculiarities of osmium deposition. It is usually said that injury to the living animal is not demonstrable for two days afterward. It is to be noted that the deposition of osmium caused by squeezing the dead brain is not in necessary opposition to the statement for the living animal. Boyce (1895) gives four days as the earliest time for commencing signs of the reaction following injury to the living animal. The earliest date of demonstrable change we have found to be associated with the vitality and species of the animal as well as its age. The size of the lesion, the temperature of the surroundings (summer or winter) and the activity and passivity of the animal, as well as the definition of "demonstrable change" all influence our judgment in this matter. Boyce tells us he noted the reaction to achieve a maximum on the tenth day in a moderately sized mammal. He gives certain criteria for the differentiation of true from false degeneration. While certain of these are not necessarily diagnostic, we give them:

1) No trace of degeneration is to be seen before fourth day of the lesion.

2) The degeneration is limited to definite anatomical groups.

3) In the case of bilateral tracts the degeneration is confined to one tract if the lesion is unilateral.

He further tells us that the staining of a healthy nerve fiber is extremely rare and may be distinguished by the straightness of its course from a truly broken up, varicose, degenerated fiber.

The frequent occurrence of occasional disperse and isolated black spots apparently without regard to anatomical systems of myelinated fibers had given us occasional cause to wonder about their origin.

Dr. Dusser de Barenne suggested to us the possibility of the migration of the Marchi drop from the original site of lesion. In order to test this, we prepared a cortical lesion and after the usual lapse of time and technic put the 4 mm. brain slices into a very strong Marchi solution. By withdrawing parts of the brain at varying times, sections were procured in which the amount of osmium penetrating the tissue had been insufficient to stain the nervous elements but had been great enough to stain the diffuse black spots referred to above. These were all found in close proximity to, and most of them within extension of, the vascular or perivascular system. The identity of many of these as phagocytic cells could be determined. We have in progress experiments designed to arrive at the origin and distribution of these elements. In ordinary Marchi preparations such spots, particularly when occurring in the tegmentum, would be interpreted as diffuse degeneration.

We have found the following procedure the most useful.

(1) The animal is killed by the use of illuminating gas at the end of 11 days for primary, and 30 days for retograde degeneration. Illuminating gas kills faster than any of the anesthetic vapors with less danger of causing changes in the nervous tissue.

(2) The preparation is bled from the heart.

(3) The brain case is opened to as wide an extent as possible without handling the brain. The dura is removed and the whole is douched or immersed in neutral formalin (formaldehyde 37%) one part, distilled water one part.

(4) This and steps 5, 6, 8 and 9, are best carried out in the dark. The tissue is removed to 3% dichromate of potassium for one week. (If it is a large brain, larger than a small rabbit, it is placed in the formalin solution mentioned above for one day and then transferred to the dichromate).

(5) The brain is now cut into parallel slices of 4 mm. thickness and returned to fresh dichromate for 2 weeks. Change whenever discolored or when precipitates form.

(6) The tissue is transferred to a mixture of 1% osmic acid solution (one part), and 3% potassium dichromate solution (four parts), for three weeks. Five volumes of fluid to one of brain is used, and it is changed whenever the osmium is depleted (determined by cautious smelling), or if the solution turns black.

(7) The sections are now washed in running water for one or two days depending upon their size.

(8) Complete dehydration is carried out over a period of time depending upon the size of the brain.

(9) Celloidin infiltration is brought about by the pressure method of Potter as given in a previous paper (Mettler, 1932).

(10) The blocks are hardened in the air in greased paper forms and stored in 70% alcohol.

(11) The sections are cut at 40 to 50 microns, cleared in chloroform and mounted in chloroform balsam.

Among the expositions and modifications of the Marchi method, in addition to those mentioned above, may be cited those of Askanazy (1897), Pollack (1898), Busch (1898), and Hamilton (1897). Busch uses a solution of 1% osmic acid, one part, and 1.5% sodium iodate, two parts, in place of the ordinary Marchi solution. Sodium iodate also replaces potassium dichromate as the preliminary solution. By such a method he avoids the yellowish color of the usual Marchi preparation, facilitates counter staining and enhances the chances of photographic reproduction. Sodium iodate in this usage has given accepted results (Collier and Buzzard, 1901), (Beevor and Horsley, 1902). Hamilton (1897) stains the sections with the Marchi mixture after sectioning.

Hamilton's procedure is:

(1) Harden in Müller's (very large brains must be injected with it) for 3 or 4 months.

(2) Do not wash—dehydrate and embed in celloidin (long stays do not seem to injure).

(3) Cut sections and store in dilute alcohol.

(4) Enclose each section in a double layer of celloidin. (Each section is arranged on a slide already covered with a firm, but not dry, layer of celloidin. The section is then covered with another layer which is allowed to become firm, not dry. Numbers may be included between the celloidin layers). Immerse in methyl alcohol in a flat dish before the section is placed on the first celloidin layer. The alcohol is taken up with lintless blotting paper, or with a blotter covered with tissue before pouring the second celloidin layer. Strip off the slide after replacing in methyl alcohol. Clip the section.

(5) A brain which has been in Müller's at least three months is pounded up in a mortar to thin creamlike consistency by the addition of Müller's fluid (preferably that in which it was hardened). Half a kilo of brain should be used to a liter of Müller's. Filter and use by mixing 200 cc. of the filtrate with 1 cc. of 1% solution of osmic acid. Place in an air-tight bottle with the sections. (Use a considerable excess) and keep at body temperature for 24 hours. Remove and wash for a few moments in water. Clean bottle thoroly.

Introduce

Pyrogallic acid	1 gram	Solution in considerable excess
Glycerin	50 cc.	
Absolute alcohol	50 cc.	
H_2O to make	400 cc.	

Put in bottle and put back for 24 hours in incubator. Wash bottle and sections again thoroly. Leave overnight in water. Fill bottle with ¼% potassium permanganate. Remove stopper and leave at room temperature for 24 hours. Remove and wash.

Use sulfurous acid to decolorize. (Strength does not matter). We have used potassium sulfite in this place. After washing, repeat the permanganate procedure and sulfurous acid treatment twice more (3 times in all).

Return to reducing fluid in incubator for 24 hours.

Wash thoroly in water, dehydrate, clarify (oil of cloves) mount in dammar lac.

It will be immediately obvious that such a procedure enables us to alternate a Marchi, Weigert for detail, and Weigert-Pal with counter stains for cells.

The sections may easily have the dichromate removed and may be stained for the cellular constituents. Staining of the cells may also be accomplished without removing the dichromate.

The chief value of this stain as we have found it has been in staining tissues which had been insufficiently impregnated by the Marchi fluid. Its time-consuming manipulations will doubtless continue to depress its popularity as a routine measure.

The use of osmium by virtue of its properties, extends to demonstration of the degeneration of peripheral nerves (Ranvier, 1878), fixation of various tissues (Ranvier, 1882) staining of peripheral myelinated material (Lenhossek, 1892), and in conjunction with other reagents in neurological stains Berkeley (1892). The handbooks by Lee (1913) and Hardesty (1902) both contain information on the use of osmium.

Other stains are occasionally employed in studies of fiber degeneration such as that of Beer (1892) and the ordinary fat stains, sudan IV, (scarlet red) (Michaelis), sudan III (Daddi), oil red O (sudan II), indophenol, (a blue stain acting like the sudan stains) and nile blue sulfate (Lorrain Smith) (see Conn, 1925), altho the best for degeneration products is probably Herxheimer's scarlet red stain as used in Alzheimer's laboratory.

Counterstains as van Gieson's, carbol-fuchsin or safranin are occasionally used following the Marchi technic to bring out cell bodies.

Since the Marchi method becomes without value after more than three months have elapsed subsequent to the occurrence of the lesion, other methods must be resorted to in delineating the myelin sequellae of traumatism. Tho in the case of peripheral nerves, regeneration of the myelin may occur, there is no doubt that in the central nervous system permanent changes remain which are demonstrable by methods of the nature of the Weigert. There is, however, another important function of such technics, in that a well prepared Weigert (1884) preparation of corresponding normal tissue is an indispensable control of the Marchi method. It sometimes happens that one has considerable cause to suspect an artifact in regions of poor myelina-

tion. Consultation of a Weigert preparation will often inform as to the correct interpretation to be made.

ALLEN, W. F. 1923. Tractus solitarius in the guinea pig. J. Compar. Neurol. **35,** 171.

ASKANAZY. 1897. Bemerkungen zur Marchi'schen Färbung und Markscheiden—Färbung nach Weigert. Centbl. Allg. Path., usw. **8,** 615.

BEER, T. 1892. Über die Verwendbarkeit der Eisenchloriddinitroresorcinfärbung für des Studium der Degeneration Peripherer Nerven. Arb. Inst. Anat. & Physiol. d. Centralnervensystems, Wiener Univ., **1892,** 53–72.

BEEVOR & HORSLEY. 1902. On the Pallio-Tectal system of fibers. Brain, **25,** 436.

BERKELEY, H. J. 1892. Die Osmium-Kupfer-Hämatoxylin-Farbung. Eine schnelle Weigert-Methode. Neurol. Centbl. **11,** 270–72.

BOYCE, RUBERT. 1895. A Contribution to the study of descending degeneration, Phil. Trans. Roy. Soc., **186,** B, 322.

BUSCH, C. K. 1898. Über eine Färbungsmethode secundärer Degeneration des Nervensystems mit Osmiumsäure—Neurol. Centbl., **17,** 476.

CIAGLINSKI. 1896. Lange sensible Bahnen in der Grauen Substanz des Rückenmarkes und ihre experimentelle Degeneration. Neurol. Centbl., **15,** 773.

COLASSAK, R. 1898. Die Herkunft des Myelins. Arch. Entwicklungsmech. d. Organ, **6,** 453–93.

COLLIER & BUZZARD. 1901. Descending mesencephalic tracts. Brain, **24,** 177 (see under Bouche's modification).

CONN, H. J. 1925. Biological Stains. Commiss. on Stand. of Biol. Stains, Geneva, N. Y.

HAMILTON. 1897. On a method of demonstrating secondary degenerations of the nervous system by means of perosmic acid. Brain, **20,** 180.

HARDESTY, I. 1902. Neurological technique. Univ. Chicago Press, Chicago.

HEILBRONNER. 1898. Rückenmarksveränderungen bei multipler Neuritis der Trinker. Hallische Habilitationschrift. Karger, Berlin.

HELLER. 1898. Zur Technik der Osmirung des Centralnervensystems. Arch. Psychiat. & Nervenkrankh, **30,** 173–175.

HOCHE, A. 1896. Über secundäre Degeneration, speciell des Gowersschen Bündels, usw. Arch. Psychiat. & Nervenkrankh, **28,** 510.

KINGSBURY and JOHANNSEN. 1927. Histological technique. John Wiley and Son. New York.

LANGLEY, J. N. and ANDERSON, H. K. 1899. Modification of Marchi's method of staining degenerating nerve fibers, J. Physiol., **24,** 31.

LEE, ARTHUR B. 1913. Microtomist's Vade-Mecum. Blakiston's, Philadelphia. See p. 373 ff.

LENHOSSEK, M. V. 1892. Der feinere Bau des Nervensystems. Fortschr. d. Med., **10,** 571–84.

LUITHLEU and SORGO. 1898. Zur Färbung der Ganglienzellen. Neurol. Centbl., **17,** 640.

MALLORY and WRIGHT. 1924. Pathological Technique, Saunders, Philadelphia.

MARCHI and ALGERI. 1897. Rivista sperimentale di freniatria e medicina legale, Vol. **12,** 3.

MCCLUNG, C. E. 1929. Handbook of microscopical technique. Hoeber, New York.

METTLER, F. A. 1932. Connections of the auditory cortex of the cat. J. Comp. Neurol. **55,** 1.

ORR. 1900. A method of staining medullated fibers. J. Path. and Bact., **6,** 287.

PARSONS, J. HERBERT. 1902. Degenerations following lesions of the retina in monkeys. Brain, **25**, part III, 24.

PELLIZZI. 1895. Sur les degenerescences secondaires dans le système nerveaux central. Arch., Ital. de Biol., **24**, 89.

POLLACK. 1898. Die Färbetechnik des Nervensystems. *Auf.* **2**. Karger, Berlin.

RANVIER, LOUIS. 1878. Lecons sur l'Histologie du Système Nerveux. 2 vols. Savy, Paris.

1822. Traité technique d'Histologie. Savy, Paris.

ROSIN, H. 1896. Ein Beitrag zur Lehre vom Bau der Ganglienzellen—Deutsche Med. Wochnsch., **22**, 495.

SINGER and MÜNZER. 1898. Beiträge zur Kenntniss der Sehnervenkreuzung. Denkschr. d. Akad. d. Wissensch., Math-Naturw. Classe, **55**, 163.

STARLINGER, J. 1899. Zur Marchi-Behandlung. Zts. Wis. Mikr., **16**, 179.

STRANSKY, ERWIN. 1903. Bermerkungen über die bei Marchi-Färbung auftretenden arteficiellen Schwärzungen. Neurol. Centbl., **22**, No. 14, 658.

TELJATNIK. 1897. Zur Technik der Marchi'schen Färbung des Centralnervensystems. Neurol. Centrbl., **16**, 521.

WEIGERT, CARL. 1884. Ausführliche Beschreibung der in No. 2. dieser Zeitschrift erwähnten Färbungsmethode für des Centralnervensystem. Ertsch. d. Med., **2**, 190.

WEIGERT, C. 1897. Die histologische Technik des Centralnervensystems, III. Ergebnisse der Anatomie und Entwickelungsgeschickte. Anat. Hefte, **7**, 3.

WLASSAK. 1898. Die Herkunft des myelins. Arch. Enwickelungs-mechanik d. Organismen., **6**, 453.

NOTES ON TECHNIC

FURTHER NOTES ON THE USE OF GENTIAN VIOLET AS A SOMATIC CHROMOSOME STAIN.—Since the appearance of the original note,[1] two samples of new batches of "gentian violet" have been extensively experimented with.

"Gentian violet," Code No. CB-8, from Coleman and Bell, is, in our opinion, the most satisfactory batch we have yet encountered. The stain afforded by this batch leaves nothing to be desired, unless an occasional technician might desire a greater intensity.

"Gentian violet," Code No. NC-13, from the National Aniline and Chemical Co., gave the most intensely purple stain of any of the various violets with which we have experimented. In the early trials, we employed plant material in which the chromosomes were large and the cytoplasm scanty, and it was not until species with many very small chromosomes and rather dense cytoplasm were stained that a serious fault appeared, namely, the deposition of tiny particles in the cytoplasm. If the sections are in the least loosened from the slip, the trouble is aggravated. Others have written us that they have had the same trouble with numerous batches of stains from different manufacturers; the concensus of opinion is that the source is some foreign substance added to the dye itself. Others beside ourselves have done much experimenting to obviate this difficulty, but no solution has yet appeared, save in manufacturing methods. Consequently, we cannot give this batch a blanket recommendation for use in the picro-alcohol method, altho at first it seemed possible to do so. The depth of the stain is precisely that which we have been striving for, but the deposits spoil everything.—DONALD A. JOHANSEN, Stanford University, Cal.

BUFFER SOLUTIONS IN STAINING PROCEDURES. A CORRECTION.—Attention has been called to a serious error in a paper[2] published in this JOURNAL in 1930 giving the formulae of buffer solutions to be used in certain staining procedures such as blood staining.

The error is one of transposition of column headings and at this late date it is hard to tell how the error occurred. It was not in the original notes from which the manuscript was prepared, and whether the

[1]Johansen, D. A. A new method of differentiating gentian violet when used as a somatic chromosome stain. Stain Techn., **7**, 17–20. 1932.

[2]French, R. W. Practical Methods for the Control of Hydrogen-ion Concentration in Staining Procedures—The use of "Buffers". Stain. Techn. **5**, 87–90. 1930

transposition took place in copying the manuscript or in type setting, it is difficult to say. As the error is serious, the table is hereby reprinted with proper corrections made. It is hoped that those who have files of the JOURNAL will see that the corrections are indicated on the page where the original article occurred.—R. W. FRENCH. Washington, D. C.

PHOSPHATE BUFFER SOLUTIONS FOR USE IN STAINING PROCEDURES

pH	$Na_2HPO_4+2H_2O$	KH_2PO_4
5.3	0.227 grams	11.583 grams
5.6	0.454	11.286
5.9	0.908	10.692
6.2	1.816	9.504
6.5	2.723	8.316
6.8	4.539	5.940
7.0	5.447	4.752
7.2	6.355	3.564
7.4	7.262	2.376
7.7	8.170	1.188
8.0	8.624	0.594

LABORATORY HINTS FROM THE LITERATURE

Under this heading are given interesting new methods in microscopy that have been noticed in other publications. The readers' assistance is urged in calling the editor's attention to any outstanding methods in this field that are overlooked. Thru coöperation with the Wistar Institute of Anatomy and Biology of Philadelphia, these same abstracts are to appear on the Laboratory Service Cards of that institute. Those desiring some of these cards without subscribing for the entire Bibliographic Service of the Wistar Institute, can order direct from STAIN TECHNOLOGY, at a price of 6 cents each.

BELEZKY, W. K. Die Pyridinsodamethode zur Imprägnation der Mesoglia (Hortegazellen, Oligodendroglia, Drenagzellen) und Retinoculoendothelzellen (für Gelatin und Celloidinschnitte). Virch. Arch. Path. Anat., **282**, 214–1224. 1931.

The method described has been worked out for the impregnation of mesoglia of the normal brain of children and embryos, and also for reticuloendothelial cells outside of the central nervous system.

For gelatin sections fix fresh material in 5 to 10% formalin; wash under tap 24 hours. Transfer to 24% gelatin in 10% phenol for 2 to 3 hours at 37° C in a covered watch glass. Cool for not over 30 minutes. Cut small pieces around the borders and fix 2 to 5 days, preferably in 10% neutral formalin, or else in 10% acid formalin, (pH 1.6 to 1.7), altho the latter may swell the gelatin excessively. If necessary, pass thru an acid solution after fixation in neutral formalin. Cut frozen sections 10 to 25 μ thick, and transfer thru a few changes of distilled water.

Prepare the following solution: to equal parts of normal $AgNO_3$ and normal Na_2CO_3 in a watch glass, add enough pyridin to dissolve the precipitate (about 50 drops). The resulting reaction is about pH 9.4; for embryonic tissue better results are obtained with a less alkaline reagent. It may be necessary to dilute the whole considerably. Impregnate for a few seconds to 2 minutes or more if dilute solutions are used. Pass thru distilled water and transfer to 10% neutral formalin. Do not agitate the containers for the blackening should proceed slowly (5 minutes). If the blackening is too rapid add acid instead of the neutral formalin. Rinse in water and examine wet. Transfer to an albumin smeared slide; if satisfactory, dry and mount.

A modification of the method for celloidin embedding is also given.

BROWN, J. H. and BRENN, LENA. A method for the differential staining of Gram-positive and Gram-negative bacteria in tissue sections. Bul. Johns Hopkins Hosp., **48**, 69–73. 1931.

Paraffin sections are stained 2 to 5 minutes in freshly filtered alum-hematoxylin (Harris). They are then washed in acid alcohol (3% HCl in 95% alcohol) until light pink, in ammonia water (1% aqua ammon. in 100 cc. water) until blue, and finally in water. Stain 2 minutes in freshly prepared alkaline gentian violet (5 drops of 5% aqu. $NaHCO_3$, with 0.5% phenol, added to 0.75 cc. of 1% aqu. gentian violet). Wash with water and apply Lugol's iodine solution for 1 minute. Wash with water, and blot. Stain 5 minutes with 0.005% aqu. rosanilin hydrochloride. Wash in water; blot but do not allow section to dry. Pass thru acetone. Place on the slide 0.1% picric acid until section becomes yellowish pink; this is a critical point and the process should be carried out with the slide over a white plate or dish. Pass successively thru acetone, acetone and xylol in equal parts, and xylol. Mount in balsam.

Cell nuclei are dark reddish-brown; cytoplasm yellowish; Gram-positive bacteria deep violet or almost black; Gram-negative bacteria bright red. Cytoplasm of leucocytes is yellowish, basophilic granules red; red blood cells yellow to red; cartilage pink.

Source or dye contents of dyes are not given. Chemical nature is given only in the case of rosanilin hydrochloride. Even in this instance the author states that

"basic fuchsin" may be substituted for it; presumably the sample used under the latter name was not labeled by the manufacturer so as to show whether it was rosanilin, pararosanilin, or a mixture of the two.

GNANAMUTHU, G. P. Note on picro-congo-red staining. J. Royal Micro. Soc., **51**, 401–402. 1931.

Picro-congo-red proved to be an excellent counterstain for Ehrlich's hematoxylin with tongue sections after Bouin fixation. Proceed as follows: shake 12.5 cc. of saturated picric acid with an equal volume of ammonia, dissolve 0.6 grams of congo red (Grübler's used), and boil off excess ammonia. Let stand until a sediment appears; dissolve this in distilled water, and add some ammonia to provide an alkaline medium.

After the sections have been stained for about 5 minutes in hematoxylin, rinse in water and counterstain with the picric congo red for 1 to 2 minutes. Rinse, dehydrate, clear and mount. The stain acts quickly and should be watched under the microscope.

Results: Hematoxylin differentiates the tendon, the fasciae of muscles, glands, cartilage, nerves, and nuclei of blood cells, while the picro-congo-red stains the muscles, articular or transitional cartilage, and the cytoplasm of red corpuscles. As in the tongue, the former tissues are scattered among and around the muscles which form the chief part of this organ, therefore this stain proves very useful.

HUNTOON, F. M. Glycerin as an adjuvant to bacterial dyes. Am. J. Clin. Path., **1**, 317–319. 1931.

Glycerin is recommended as an adjuvant to the usual dye solutions employed for bacterial staining in working laboratories because it aids in the keeping properties of these solutions and because it aids in the staining, greatly increasing the clearness of the microscopical picture.

For Gram staining, glycerin crystal violet is used: mix 15 parts of 3% solution of crystal violet in 95% alcohol with 85 parts of 30% aqueous solution of glycerin. Counterstains used are: A. *Red*. Mix 10 cc. of carbol-fuchsin with 100 cc. of a 25% aqueous solution of glycerin. B. *Brown-yellow*. Shake up 2 grams Bismarck brown in 100 cc. of water and filter. To the filtrate add 30 to 40 cc. of glycerin and mix. Standard Gram's iodine solution and an acetone alcohol decolorizer (75 parts alcohol to 25 parts acetone) are employed. The technic of staining is that of the standard Gram.

For staining tubercle bacilli in sputum two methods are suggested: (1) the above crystal violet solution may be used, steaming one to two minutes or longer; if accidentally boiled, the stain does not precipitate. Wash in water, decolorize by any of the standard methods, wash, and counterstain with Bismarck brown. (2) glycerin carbol-fuchsin (5% phenol—75 cc.; glycerin—25 cc.; saturated alcoholic solution of basic fuchsin—10 cc.) and glycerin methylene blue (25% aqueous solution of glycerin—100 cc.; 1% solution of sodium hydrate—1 cc.; saturated alcoholic solution of methylene blue—10 cc.) may be employed to give the usual red-blue picture.

All these dye solutions keep well.—*M. Sherago.*

KISSER, J. Neue Erfahrungen auf dem Gebiete des Schneidens harter Objekte. Zts. Wis. Mikr., **48**, 320–342. 1931.

Several procedures for softening woody tissues are given. Water can be used; but if cell content has to be preserved, very little heat, or better none at all, should be applied. If cell content does not matter, the material may be boiled in dilute glycerin, or exposed to steam. The wood is then transferred to a mixture of water, alcohol, and glycerin, and left therein until it reaches the desired consistency. For very hard material hot water or steam may be applied during cutting.

Diaphanol (chloridioxide-acetic-acid) and chloridioxide in nitric acid (Niesmann), are recommended. The author finds that H_2O_2 is a good agent also. The most suitable of all media, however, remains HF. It has been used in 20% and 50% solutions and at full strength. Both alcoholic and aqueous solutions are used, the alcoholic offering some advantage when the tissue consists of hard and soft elements. Phenol may find broader application—it proved very useful for

lignite. Hot alcohol under pressure is helpful for hard tissue, (Jeffrey). The temperature may be lowered, however, to 70° to 80° C, (instead of 160°), if prolonged. Acetone softens in a similar way.

Celloidin remains the best embedding medium, and according to Jeffrey, pressure has to be applied.

La Cour, L. Improvement in every day technic in plant cytology. J. Royal Micro. Soc., **51**, 119–126. 1931.

Methods are discussed for killing and fixing root tips, and pollen mother cells within the buds or preferably in smears. Three new fixatives, denoted 2 B, 2 BE and 2 BD are recommended: 2B) 1% chromic acid 90 cc., potassium bichromate 1 g., sodium sulfate 0.5 g., urea 1 g., 5% acetic acid 10 cc., 2% osmic acid 15 cc., distilled water 45 cc.; 2 BE) (best for root tips, smears), 1% chromic acid 90 cc., potassium bichromate 1 gr., saponine 0.05 g., 5% acetic acid 10 cc., 2% osmic acid 15 cc., distilled water 45 cc.; 2 BD) (good for root tips, and after Carnoy) 1% chromic acid, 100 cc., 1% potassium bichromate 100 cc., saponine 0.1 g., 2% osmic acid 30 cc., 5% acetic acid 30 cc. These new fixatives have been successful with several genera of monocotyledonous and dicotyledonous plants. When buds were embedded, Carnoy's fluid was used as a sinker, preceding one of the three liquids.

Several changes of tepid water are preferred to prolonged running water. Chloroform is preferred to xylol. In certain cases, where Zirkle's butyl alcohol is unsatisfactory, chloroform may be introduced between this alcohol and paraffin.

Gentian violet is preferred to Heidenhain's hematoxylin. With this stain, due to the transparency of chromosomes, even very heavy sections present clear pictures. Some violets are stated to fade; (no source of dye used is mentioned, however.) If gentian violet, post-treated with iodine-potassium-iodide proves unsatisfactory, the following treatment may be advantageous: Stain in 1% gentian violet for 10 minutes, (3 hours if material has been fixed in Carnoy); rinse in water; absolute alcohol 2 seconds; iodine-iodide, in 80% alcohol, 2 minutes; absolute alcohol, 2 seconds; 1% chromic acid, 15 seconds; absolute alcohol, 10 to 15 seconds; clove oil; xylol.

Maneval, W. E. The staining of flagella of bacteria, with special reference to mordants. J. Bact., **21**, 313–321. 1931.

The writer investigates several mordants and gives formulae for 24 which give satisfactory results in flagella staining. All the formulae contain 20% of 10% tannic acid or 10% of this tannic acid solution plus 10% colloidal silver (a 1% silver nitrate solution to which NH_4OH is added until precipitate just dissolves). All mordants contain 10 drops of 10% alcoholic fuchsin. They further contain $AlCl_3$, $CuCl_2$, $FeCl_3$, $Fe(OH)_3$ Ni Cl, or $ZnCl_2$, separately or in combination. The writer implies that there is little choice between these various mordants.

The most satisfactory stain proved to be an anilin-alcohol-fuchsin: distilled water 30 cc., 10% alc. sol. basic fuchsin 10 cc., anilin oil and alcohol (1:3) 5 cc., 4% acetic acid 1 cc. The air-dried slides are placed on a level surface, treated with 4 to 8 drops of mordant for 2 to 10 minutes; are washed in 6 to 10 changes of water; after carefully draining 3 to 5 drops of stain are applied for 3 to 5 minutes. They are then washed thoroly and dried.

The source, dye-content, or chemical nature of the dyes used is not given. The writer states, moreover, that rosanilin or methyl violet may be substituted for basic fuchsin; this suggests either that the basic fuchsin employed under that name is not rosanilin or that the author is ignorant as to its chemical composition.

Martinez Pérez, M. R. Contribution à l'étude des terminaisons nerveuses dans la peau de la main. Trav. Lab. Rech. Biol. Madrid **27**, 187–226. 1931. (Abs. in Zts. Wis. Mikr. **48**, 384).

The following procedure is offered for the demonstration of nerve terminations and Meissner's bodies in human skin: Place slices 3 to 4 mm. thick in a 10 to 15% aqueous solution of chloral hydrate for 24 hours. Rinse in distilled water and fix in absolute alcohol for 24 hours, add 4 drops of ammonia to every 60 cc. of alcohol. Rinse in distilled water until the objects sink. Transfer to a 1.5% solution of silver nitrate for 7 to 8 days at 37° C. Reduce in formol pyrogallic acid for 24 hours.

SCHMUCK, M. L. and METZ, C. W. A method for the study of chromosomes in entire insect eggs. Science, **74**, 600–601. 1931.

By means of this method chromosomes are revealed in entire insect eggs without staining the yolk or other substances. Intact mitotic figures may be studied from various, most suitable angles. This is accomplished by rotating the eggs to the desired position.

Fix in modified Carnoy's fluid, (equal parts of chloroform, absolute alcohol, and glacial acetic), for ½ to 2 hours. Wash in several changes of absolute alcohol for about 2 hours. Pass thru 95%, 85%, and 70% alcohols respectively (1 hour each), and thru 50%, 30%, and 15% alcohol (30 minutes each); then wash in several changes of water. Place in cold normal HCl for 15 minutes, and transfer to a similar solution heated to 60°C for 8 to 10 minutes. Rinse in the cold HCl, and place in SO_2-water for 2 to 5 minutes. Stain in fuchsin-sulfurous acid for 1 hour, (See Lee's Microscopists Vade mecum, 9th ed. pp. 437–438, and footnote p. 438). Wash in 2 changes of SO_2 water, and rinse in several of tap water.

The HCl and SO_2 solutions should be fresh and the fuchsin sulfurous acid solution should be an amber color. Dehydrate rapidly, clear in xylol and mount in balsam as usual, or between cover slips. In rolling the eggs during observation the cover glasses are moved to and fro, and this suffices to turn them. For more careful manipulation examine the eggs in immersion oil.

Good results have thus been obtained with eggs of the fungus gnat—*Sciara coprophila* Lint. Their size is 0.2 mm in length and 0.1 mm in thickness. The procedure would probably work with other and larger eggs also.

SKUPIENSKI, F. X. Influence du rouge neutre sur le dévelopment de certains Myxomycètes. Acta Soc. Bot. Polon., **8**, No. 3/4, 1931.

Neutral red, quite harmless when added to the medium after its sterilization, may inhibit the normal development of certain Myxomycetes when the stain is sterilized with the medium. Four fungi were grown on normal media, on media with neutral red added after sterilization and with neutral red sterilized within the medium.

If neutral red is added after sterilization *Dictyostelium mycoroides* stands a 1:15000 dilution, *Didynium nigripes* and *Polysphondylium pallidum* develop normally at a dilution of 1:5000, and *Polysphondylium violaceum* goes thru the usual life cycle at a dilution of 1:2000.

When neutral red is sterilized with the medium in a dilution of 1:15000, *Didynium nigripes* develops poorly and fructifications if present, are somewhat abnormal; *Polysphondylium pallidum* developed hardly any sporangia; *Polysphondylium violaceum* develops normally, except that the fructifications are of shorter stature than otherwise, with dark violet instead of light purple sporangia. Other fungi, which are normally nearly colorless, show various shades of pink. *Dictyostelium mycoroides* fails to develop under these conditions.

The toxic influence of neutral red after a triple sterilization (at 100°C) is evident. It is of interest to note that if these fungi are first grown on media containing the stain, added after unsterilization, they may be transferred to the medium sterilized with neutral red and develop all the stages of their usual life cycle.